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Chronic Lithium Treatment Decreases Neuronal Activity in the Nucleus Accumbens and Cingulate Cortex of the Rat

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Although the efficacy of lithium as a mood stabilizer is well documented, the mechanism of its therapeutic effect associated with prolonged treatment remains unknown. Identifying discrete brain regions and neural pathways that are functionally altered following long-term lithium treatment is central to elucidating a psychotherapeutic mechanism. We have used a sensitive and quantitative histochemical assay for the determination of cytochrome oxidase (CO) activity, a mitochondrial marker of neuronal activity, to determine the effect of repeated lithium treatment on regional neuronal activity in the rat brain. Oral lithium treatment (21 days) selectively decreased cytochrome oxidase activity in the cingulate cortex and regions of the nucleus accumbens. These decreases were not seen after 5 days of lithium administration, although serum lithium concentrations were similar after both 5 and 21 days of treatment. The analysis of interregional correlations further suggests a role for amygdala pathways in the effects of lithium following 21 days of treatment. The implications of these data for understanding the mechanisms of action of lithium are discussed. ***[Neuropsychopharmacology 21:229–237, 1999]*** *© 1999 American College of Neuropsychopharmacology. Published by Elsevier Science Inc.*

KEY WORDS: *Lithium; Cytochrome oxidase; Cingulate cortex; Nucleus accumbens; Amygdala; Imaging; Bipolar disorder*

Lithium is an effective psychotherapeutic drug. It is the drug of choice for the acute treatment and long-term prevention of mania and the prophylaxis of bipolar disorder. The influence of lithium on postreceptor mechanisms of signal transduction are well documented (Manji and Lenox 1994; Manji et al. 1995 for reviews). However, the relationship of these neurochemical and molecular actions to the mood stabilizing effects of lithium seen after prolonged treatment remain uncertain. An understanding of the biological basis of lithium's mood stabilizing effect could perhaps be obtained by characterizing the anatomy of neural systems functionally changed by chronic lithium administration. Such an approach is directed toward the "final common pathway" of lithium's antimanic effects and would reflect the integration of its multiple cellular and subcellular actions.

With the development of improved imaging techniques, discrete human brain regions and distributed neural circuits have been implicated as targets of mood disorders and as substrates of therapeutic drug effects. For example, the comparison by positron emission tomography (PET) of unmedicated bipolar patients and control subjects suggested an increased activity in rostral ventral cingulate cortex during the manic phase

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(Drevets et al. 1997). Studies also indicate that a possible treatment effect provided by lithium administration is to reduce neuronal activity in rat cortical regions. Chronic oral lithium treatment has been shown to reduce c-fos expression in the frontal cortex significantly (Miller and Mathe 1997). Serotonin transporters are increased throughout the cerebral cortex of rats treated chronically with lithium (Carli and Reader 1997), possibly decreasing serotonin neurotransmission in these regions. Sustained changes in synaptic activity are associated with altered ion pump activity, energy demand, and, ultimately, the amounts and activity of certain of the neuronal mitochondrial electron transport enzymes. CO, or complex IV, is the terminal enzyme of the electron transport chain and catalyzes the transfer of electrons to generate ATP via the coupled process of oxidative phosphorylation (Gonzalez-Lima 1992). CO activity is regulated by and closely correlated with neuronal functional activity (Wong-Riley, 1989). CO histochemistry has increased anatomical resolution and greater sensitivity to sustained changes in metabolic activity as compared to 2-DG autoradiography (Gonzalez-Lima 1992; Wong-Riley 1989; Hevner et al. 1993) and lesser dependence than c-fos expression on specific intracellular signaling mechanisms to transduce changes in activity. Histochemical techniques allow mapping of CO activity with excellent anatomical resolutions in the brain of many species (Brauth 1990; Hovda et al. 1992; Harley and Bielajew 1992; Gonzalez-Lima and Cada 1994). We have modified a histochemical method for the quantification of brain regional CO activity (Gonzalez-Lima and Cada 1994) to enable sensitive and reliable measurements of CO specific activity and have applied this technique to identify significant changes in neuronal activity in discrete areas of the rat brain following chronic lithium treatment. Interregional correlation was also used to generate an initial anatomical model of the neural pathways underlying lithium's gradually developing, long-term effects on brain function.

METHODS AND MATERIALS

Animals

Twenty four male Sprague–Dawley rats (250–300 g) housed four to a cage in a 14-hour dark, 10-hour light daily cycle (lights on at 7 AM), were divided into three groups. The first group received standard rat chow (Teklad 8640) containing 0.1% w/w lithium carbonate (Harlan Teklad, Madison, WI) *ad libitum* for 21 days. The second group received standard chow for 16 days and was then switched to lithium-containing chow for the last 5 days of the study. The third group received standard chow for all 21 days of the study. All rats were given free access to water, and isotonic saline (0.9%) was freely available to minimize the hyponatremic effect of lithium. All procedures were in accordance with the NIH guide for the Care and Use of Laboratory Animals (1996) and the Emory University Animal Care and Use Committee.

Serum Lithium Concentration

Serum lithium concentration was monitored once a week throughout the duration of the study. Blood was collected from tail snips made while rats were anesthetized with halothane. Upon study completion and subject decapitation, trunk blood was collected and assayed to determine final blood lithium concentration. The serum was collected by centrifugation (10,000 *g* for 10 min), and lithium concentrations were determined by flame photometry.

Tissue Collection

At study day 21, all rats were sacrificed by decapitation. Brains were quickly removed, rinsed with phosphate-buffered saline (PBS), frozen on dry ice, and stored at −80°C.

Sectioning and CO Histochemistry

Six rats were randomly selected from each group for brain sectioning. Animals that were selected from the treatment groups had serum lithium concentrations at the lower end of the range of values associated with the highest probability of therapeutic benefit (0.6 to 1.2 mm/1) in humans (Gelenberg et al. 1989). At the end of the study, serum lithium concentrations in the 5-day group and the 21-day group ranged from 0.35 to 0.43 mEq/1 and from 0.37 to 0.47 mEq/1, respectively. Thirty-μm thick coronal brain sections were cut on a cryostat and thaw-mounted onto glass slides. The sections were stored at −20°C until assayed for CO activity. Sectioning was performed within 48 hours of histochemical staining.

Quantitative CO histochemistry was performed on slide-mounted 30-μm-thick brain sections of frozen tissue using a modification of the method of Gonzalez-Lima (Gonzalez-Lima and Garrosa 1991). In an attempt to improve consistency of staining, we removed cobalt chloride from the staining solution. The addition of cobalt darkened the diaminobenzidine (DAB) reaction product, but in our experience also increased variability in the density of staining between assays. Briefly, slides were fixed by immersion in 0.05% glutaraldehyde, 10% sucrose in 0.1 M phosphate buffer (pH 7.4) for 5 min at 4°C. Slides were then washed in 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose three times for 5 min each. Slides were then incubated in an oxygenated reaction media (25 g sucrose, 10 mg catalase, 37.5 mg cyto-

chrome C, 250 mg DAB, 1.25 ml DMSO in 500 ml of 0.1 M phosphate buffer (pH 7.4)) in the dark for 60 min at 37°C. The tissue was then fixed in 10% buffered formalin, 10% sucrose for 30 min. The slides were then put in successive dehydration baths of 30, 50, 70, 90, 95, and 100% EtOH for 5 min each followed by xylene for 20 min. Finally, coverslips were mounted using DPX mounting media.

The quantification of brain regional CO activity involved reference to a set of standards of known CO specific activity included in each staining bath. CO standards were constructed from whole rat brain homogenates used either undiluted or following serial dilution with microwave-inactivated brain homogenate to yield mixtures containing 10, 20, 30, 70, 80, or 90% by weight active brain homogenate. Two grams of each standard were prepared and frozen in a cylindrical mold (10-mm diameter), and stored at −80°C. It has been previously demonstrated that the optical density (specific activity) of these histochemically stained standards is directly proportional to their thickness (Cada et al. 1995; Gonzalez-Lima and Cada 1994; Gonzalez-Pardo et al. 1996). To increase the upper range of the standard curve, two or three 30-μm thick slices of appropriate standards were stacked on top of each other. A total of 11 duplicated standard sections were added to each staining bath. Before histochemical staining, the specific activity of CO (μmol/min/mg protein) in each standard was determined by a modification of the spectrophotometric assay of Hevner et al. (1993). Briefly, 30 to 50 mg (wet weight) of each standard was homogenized using a handheld polypropylene pestle in 100 μl of buffer consisting of 0.32 M sucrose, 1 mM dipotassium EDTA, and 0.1 mM Tris. Triplicate aliquots (3.5 μl) of these tissue homogenates were collected, and their protein content was determined by the method of Lowry et al. (1951). Detergent-solubilized samples were prepared by combining 3.5 μl of tissue homogenate, 12.5 μl of 10% deoxycholic acid, and 234 μl of isolation buffer, mixing with a polyproplyene pestle, and incubating room temperature for 5 min with occasional vortex mixing. All spectrophotometric readings were carried out at 37°C using 0.07% cytochrome C as the substrate. The substrate solution was prepared from a 1% cytochrome c solution that had been previously reduced with sodium ascorbate and dialyzed against 0.05 M phosphate buffer. The spectrophotometer was zeroed using a blank consisting of 990 μl of substrate solution, 10 μl of detergent-solubilized sample, and 10 μl of 0.1 M potassium ferricyanide. Samples to be assayed were prepared by quickly mixing 990 μl of substrate solution and 10 μl of detergent-solubilized sample. Absorbance readings were taken at 500 nm every 15 s for 2 min. The formula: (Δ OD/E)/[tissue] where Δ OD = change in absorbance over 1 min; E= the difference between the molar extinction coefficients for oxidized and reduced cytochrome C (19.6); and tissue = mg protein, was used to calculate the specific activity of cytochrome oxidase in each sample.

Image Analysis

The MCID imaging software system (M2 Version 3.00, Imaging Research Inc., Ontario, Canada) was used for all image analysis. Digital images of stained sections were collected under controlled exposure, magnification, and illumination conditions using a digital black and white camera (Xillix Microimager) positioned above an adjustable light box. The relative optical density of each homogenate standard was measured, and a standard curve of mean optical density plotted against CO specific activity was constructed. The left and right hemisphere, representative of 14 brain regions of interest (Fr = frontal cortex; Cg = cingulate cortex; AcbCn = nucleus accumbens, cone; AcbSh = nucleus accumbens, shell; AcbC = nucleus accumbens, core; CPm = medial caudate putamen; CPl = lateral caudate putamen; Bl = basolateral amygdaloid nucleus; La = lateral amygdaloid nucleus; Ce = central amygdaloid nucleus; Pir = piriform cortex; C1 = hippocampus (CA1); MD = mediodorsal thalamic nucleus; LH = lateral hypothalamus), were sampled using the MCID system and using the atlas of Paxinos and Watson (1986). A given region of interest (ROI) was manually outlined based on anatomical landmarks at a consistent anterior–posterior location for each animal. The relative optical density was determined and converted, via reference to the standard curve, to a measure of CO specific activity within the region. Optical density measurements were performed by investigators blind to the treatment assignment.

Significant differences between treatment groups were analyzed using a two-way analysis of variance (ANOVA) (Statview, SAS Institute Inc., NC, USA) and inter-regional correlations of CO activity within each treatment group were defined by Pearson product-moment correlation coefficients (SAS, SAS Institute Inc., NC, USA). Significant correlation between two areas implies that the activity in these areas are related and may vary together.

RESULTS

Quantitative CO Histochemistry

The histochemical reaction product of CO activity was a dark brown staining of tissue sections and exhibited excellent anatomical delineation and a large (three- fourfold) range of optical density over the brain regions sampled. The homogenate standard curve was linear across a range of CO activity that included all values for the ROI (Figure 1). Three separate histochemical staining assays were needed to process all coronal sections

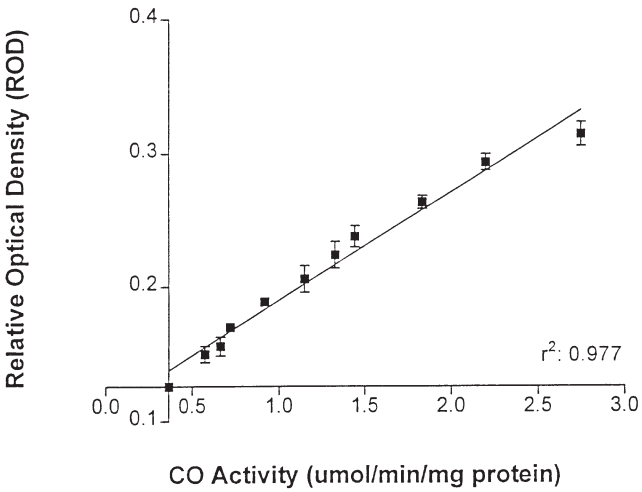

Figure 1. The composite standard curve used in analysis of these data was derived from three separate calibration curves. Relative optical density following histochemical staining (ROD) was plotted against the mean CO activity. The correlation coefficient (r^2) from a linear regression analysis of the data points is also shown.

from 18 rat brains; two animals from each treatment group were included in each assay. Comparison of standard curves for each assay indicated similar slopes and axis. Cytochrome oxidase activity within discrete brain areas of control animals demonstrated small interindividual variability (Table 1). Estimates of rat brain regional CO activity were comparable to those of prior reports (Wong-Riley 1989; Gonzalez-Lima and Cada 1994).

Effects of Lithium Treatment on Brain Regional CO Activity

There was a highly significant effect of lithium on CO activity in the rat brain that was dependent on brain area sampled ($F(2,21) = 5.5$, $p < .0001$]. Compared to the standard chow fed control group, oral lithium administration for 5 consecutive days had no significant effects on CO specific activity within any brain region examined (Table 1) ($p = .3$). However, oral lithium administration for 21 consecutive days significantly altered CO-specific activity as compared to both the control ($p < .0001$) and 5 day treated ($p < .0001$) groups. Compared to controls, CO activity in the cingulate cortex and in the core, shell, and cone subterritories of the nucleus accumbens was significantly decreased following lithium administration for 21 consecutive days (Table 1, Figures 2, 3). These effects were bilateral and region selective, because CO activity was not affected by 21 day lithium in other limbic or cerebral cortical or striatal regions. The comparison of CO activity for the left and right hemisphere indicated no significant differences for any treatment group [$F(2,21) = 0.162$, $p = .85$].

Correlation Analysis

In addition to treatment group differences in regional CO activity, we examined interregional correlations in

Table 1. CO Activity in Selected Brain Regions Following Lithium Treatment

		LEFT			RIGHT		
		Control	5 Day	21 Day	Control	5 Day	21 Day
Neocortex	Fr	1.35 ± 0.07	1.44 ± 0.04	1.47 ± 0.09	1.34 ± 0.08	1.48 ± 0.05	1.47 ± 0.06
	Cg	1.87 ± 0.11	1.80 ± 0.08	1.49 ± 0.12*	1.81 ± 0.08	1.80 ± 0.06	1.47 ± 0.14#
	Pir	1.09 ± 0.08	1.00 ± 0.01	1.18 ± 0.26	0.91 ± 0.04	1.03 ± 0.10	1.09 ± 0.02
Hippocampus	C1	1.40 ± 0.06	1.34 ± 0.06	1.41 ± 0.04	1.45 ± 0.07	1.40 ± 0.04	1.36 ± 0.06
Striatum	AcbCn	1.98 ± 0.12	1.78 ± 0.13	1.47 ± 0.09**	1.91 ± 0.10	1.76 ± 0.13	1.50 ± 0.10*
	AcbSh	1.98 ± 0.08	1.97 ± 0.15	1.42 ± 0.07***#	1.91 ± 0.06	1.96 ± 0.13	1.54 ± 0.09**#
	AcbC	1.85 ± 0.10	1.80 ± 0.12	1.29 ± 0.08**##	1.87 ± 0.10	1.83 ± 0.10	1.41 ± 0.01*#
	CPM	1.44 ± 0.17	1.56 ± 0.06	1.34 ± 0.09	1.44 ± 0.07	1.51 ± 0.03	1.39 ± 0.10
	CPI	1.47 ± 0.08	1.47 ± 0.03	1.41 ± 0.12	1.43 ± 0.05	1.45 ± 0.02	1.35 ± 0.04
Amygdala	BI	1.40 ± 0.12	1.36 ± 0.08	1.48 ± 0.13	1.36 ± 0.09	1.34 ± 0.09	1.41 ± 0.05
	La	1.09 ± 0.07	1.06 ± 0.09	1.23 ± 0.10	1.06 ± 0.04	1.14 ± 0.10	1.13 ± 0.01
	Ce	1.25 ± 0.09	1.35 ± 0.04$	1.48 ± 0.10	1.22 ± 0.09	1.17 ± 0.01	1.33 ± 0.05
Hypothalamus	LH	1.24 ± 0.08	1.30 ± 0.06	1.22 ± 0.11	1.19 ± 0.05	1.31 ± 0.04	1.30 ± 0.12
Thalamus	MD	1.37 ± 0.07	1.36 ± 0.07	1.56 ± 0.07	1.35 ± 0.06	1.44 ± 0.08	1.52 ± 0.09

Mean CO activity ± SEM for rats ($n = 6$) fed standard rat chow (control) or rat chow containing 0.1% w/w lithium carbonate for 5 or 21 days. Data are shown for the left and right hemispheres in brain regions throughout the neocortex, hippocampus, striatum, amygdala, hypothalamus, and thalamus. See method section for key to brain area abbreviations. Significant differences from control are shown by $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$. Significant differences from 5 day are shown by $^{\#}p < .05$, $^{\#\#}p < .01$. Significant differences between left and right hemispheres are shown by $^{\$}p < .05$ (ANOVA followed by Fisher's post hoc test).

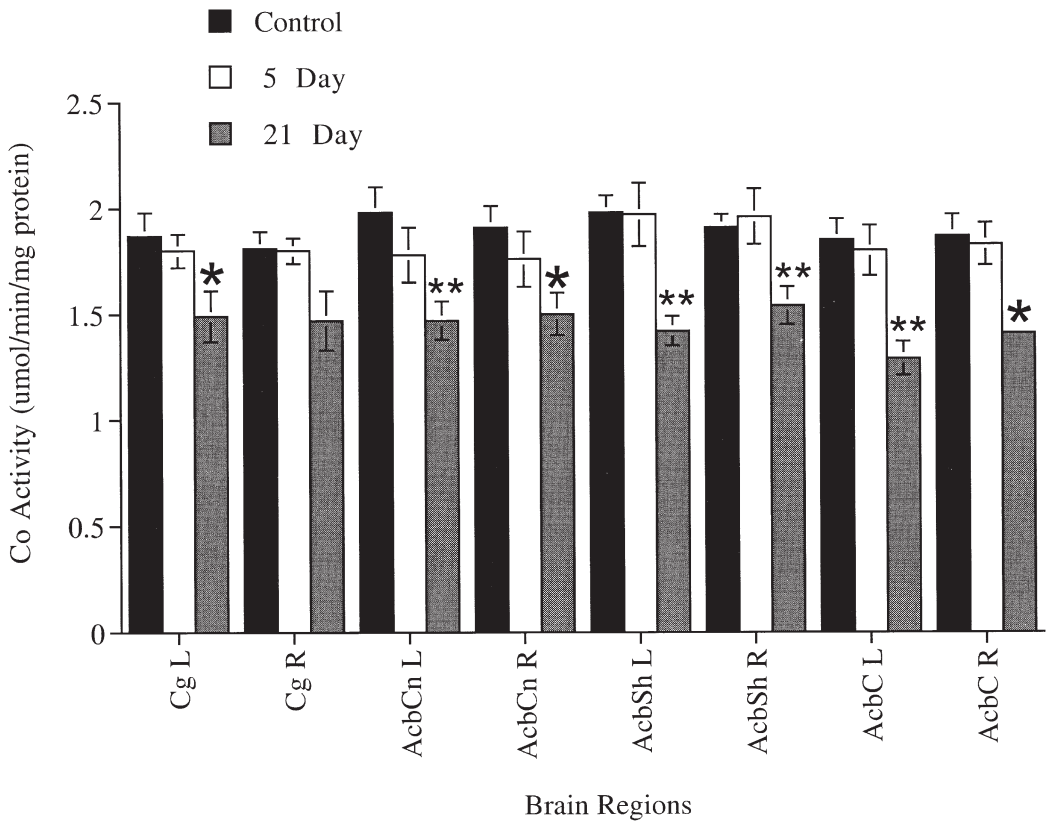

Figure 2. Histogram showing mean CO activity (± SEM) in cingulate cortex (Cg) and subterritories of the nucleus accumbens (AcbCn, AcbSh, AcbC) in rats fed standard rat chow (control) or rat chow containing 0.1% w/w lithium carbonate for 5 or 21 days. CO activity is significantly decreased in the cingulate cortex and accumbens following 21 days of lithium treatment (*$p < .05$, **$p < .01$, ***$p < .001$). Data for the left and right hemispheres are shown.

CO activity within groups (Figure 4). There were significant correlations of CO activity ($p < .01$) between accumbens sub nuclei and both the frontal cortex and medial caudate putamen in control animals (Figure 4). This pattern was altered in animals treated for 5 days with lithium by the loss of significant intra-accumbens correlation for the cone subregion and the strengthening of the relationship between the hippocampus (CA1) and core and shell subregions (Figure 4). Interregional correlations of CO activity were distinctly different following 21 days of treatment as compared to both the control and 5-day lithium treatment groups. Significant intra-amygdaloid correlations were found, and correlations were strengthened between nuclei of the amygdala and the frontal cortex (Figure 4). Twenty-one days of lithium administration resulted in new significant correlations between the cingulate cortex and both the frontal cortex and nucleus accumbens; correlations between these latter two regions and the thalamus (centromedial nucleus) were weakened. The lithium-induced alterations in interregional correlation coefficients were primarily localized to the left hemisphere.

DISCUSSION

Lithium remains the drug of choice for the treatment of acute mania and the prophylaxis of manic-depressive disorder (bipolar). The neurobiological basis of bipolar disorder has yet to be established, and the mechanism of psychotherapeutic action of lithium remains unclear. Cellular and subcellular mechanisms of action have been advanced for lithium (Carli et al. 1997; Mathe et al. 1994; Jousisto-Hanson et al. 1994; Manji and Lenox 1994; Manji et al. 1995 for reviews). The use of functional imaging techniques has the potential to identify the neural pathways affected by lithium administration and, thus, integrate the subcellular and cellular actions of lithium into an anatomical model. We have quantitatively mapped CO activity within discrete areas and nuclei of the rat brain and used these measurements to assess the effect of chronic treatment with lithium on distributed neuronal activity in the rat brain. Estimates of CO activity or expression have been used to provide functional neural explanations of neurogenesis, learning (Gonzalez-Lima 1992) and behavioral pathology

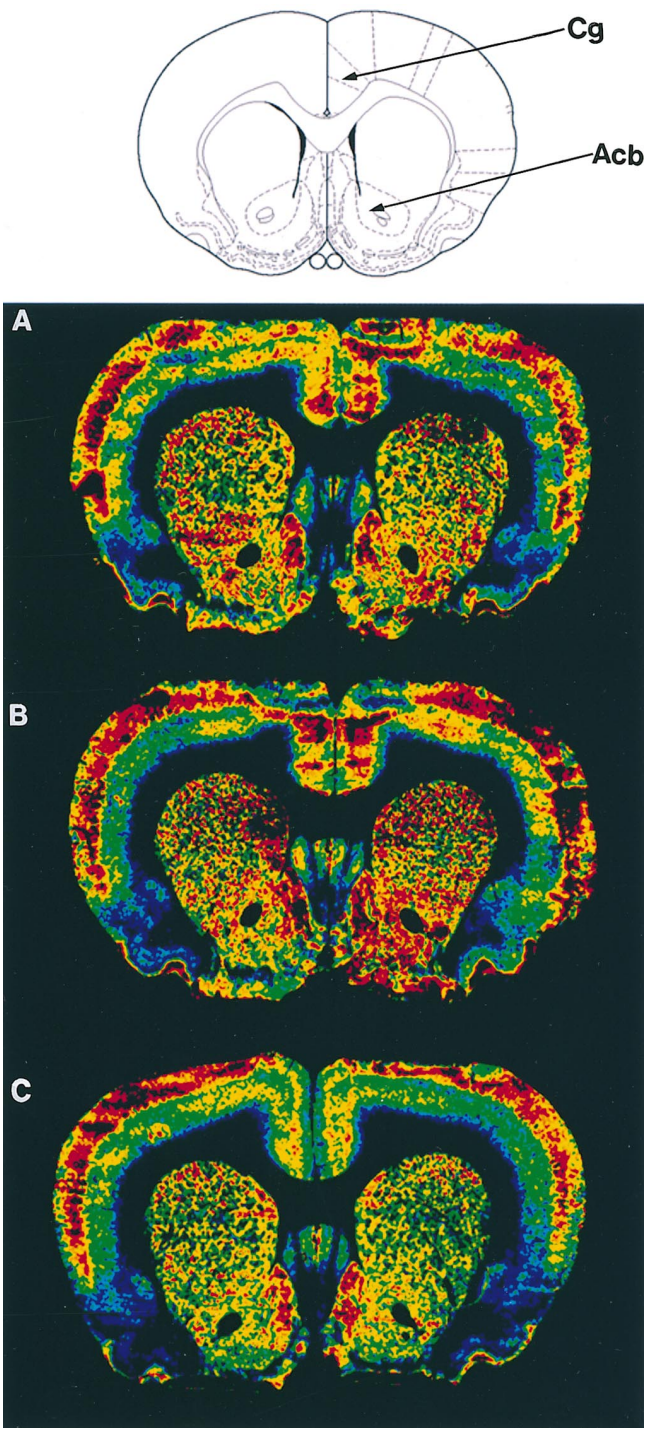

Figure 3. Digitized images (Adobe Photoshop, Adobe Systems, Inc.) of a representative coronal brain section at the level of the cingulate cortex and nucleus accumbens from a rat fed: **(A)** standard chow; **(B)** chow containing 0.1% w/w lithium carbonate for 5 days; **(C)** chow containing 0.1% lithium carbonate for 21 days. The images have been color-rendered based on staining density with areas exhibiting the highest CO activity shown in red and areas exhibiting the lowest CO activity shown in blue. Above the images is a line drawing taken from the rat atlas of Paxinos and Watson to illustrate the approximate anterior–posterior location of the sections shown (bregma 1.2 mm); Cg = cingulate cortex.

(Simonian and Hyman 1995). To our knowledge, this is the first report of the use of CO brain maps to explore the neural pathways involved in the pharmacotherapy of psychiatric disorders.

The results of this study demonstrate that oral administration of lithium for 21 consecutive days significantly decreased CO activity in the anterior cingulate cortex and areas of the nucleus accumbens as compared to untreated controls. Neither cingulate nor nucleus accumbens CO activity was altered following 5 days of lithium treatment. This time course of lithium's effect on CO activity in accumbens and cingulate cortex parallels the gradual course of onset of the therapeutic effect of lithium in patients. Other studies in the rat examining the cellular and subcellular mechanisms of action of lithium in the cortex have found no effect of short-term (2–6 days) lithium treatment as compared to significant effects following long-term (3–4 weeks) treatment (Nestler et al. 1995; Carli and Reader 1997). Interestingly, the same brain regions affected here by 3 weeks of lithium treatment have been implicated in mood disorders and in the therapeutic effects of lithium in imaging studies in humans. Images of human regional cerebral blood flow (rCBF) and glucose metabolism as correlates of synaptic activity, have preliminarily identified areas of abnormally increased activity in regions of the cortex during the manic phase (Drevets et al. 1997). This increase in neuronal activity was more clearly demonstrated in a positron emission tomography (PET) study where bipolar patients were withdrawn from lithium treatment. Lithium withdrawal was associated with the development of manic symptoms and a significant increase in rCBF in the anterior cingulate cortex (Goodwin et al. 1997). These data suggest that during mania, synaptic activity is increased within cortical areas, particularly the anterior cingulate, and that suppression of this overactivity may be important to the antimanic effects of lithium. The findings of this study support the cingulate cortex as a site of action of lithium's mood stabilizing effects. A major direct innervation of the nucleus accumbens emanates from the cingulate cortex (Alexander et al. 1990, Devinsky et al. 1995). The cingulate cortex and nucleus accumbens are components of an anatomically defined basal ganglia–thalamocortical circuit (Alexander et al. 1990). This circuit is considered to be a key element of the functional organization of the limbic system and is often implicated in mood regulation (Groenewegen et al. 1996). It would seem reasonable that regions within this circuit may be functionally altered by lithium administration as part of the mechanism of action of its mood stabilizing effect.

Evidence from both experimental animal and human studies indicate that emotional behavior is critically regulated by the amygdala (Lukaszewska et al. 1980; Frank and Stutz 1982; LeDoux 1992; Kalynchuk et al.

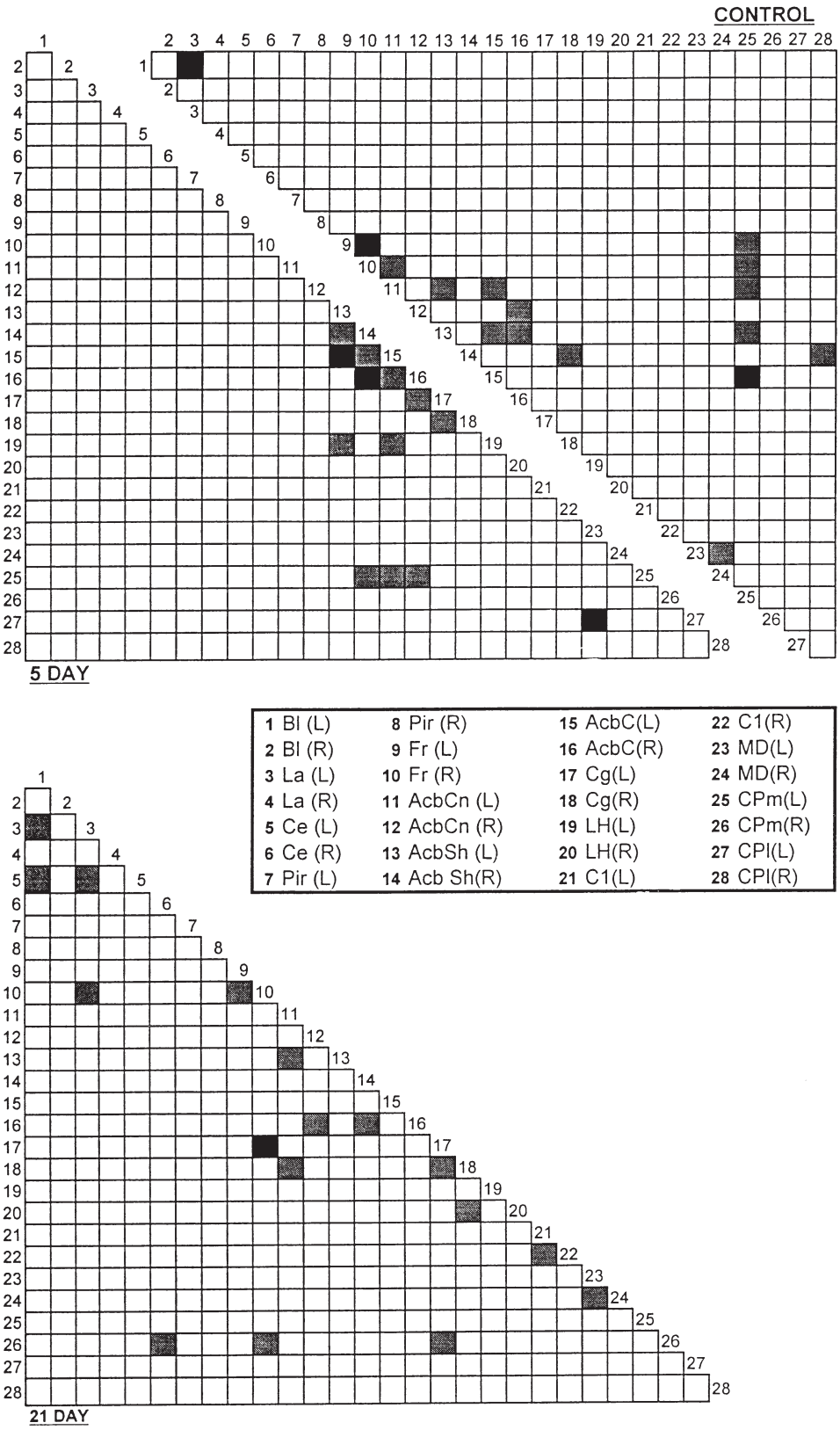

Figure 4. Matrices illustrating significant inter-regional correlations (Pearson product moment correlations) of CO activity. A matrix is shown for each treatment group [standard chow (control), chow + lithium for 5 days (5 day), chow + lithium for 21 days (21 day)], and significance of correlations is represented by degree of shading (unshaded = $p > .01$); gray = $p < .01$; black = $p < .001$). See method section for key to brain area abbreviations.

1998; Lane et al. 1997; Reiman et al. 1997). This study found no significant effect of either 5 or 21 days of lithium administration on CO activity in any of the nuclear groups of the amygdaloid complex. However, when these data were examined for significant within-group correlations of CO activity between brain regions, an effect of lithium treatment on amygdaloid activity was suggested. Because discrete brain regions do not function as individual entities but within a network of complex interactive circuitry, it may be informative to compare CO activity as interregional correlation matrices between treatment groups. In this way, an initial anatomical model of neural pathways affected functionally by lithium can be constructed. Interregional covariance or correlation matrix represents a starting point in network analysis (Horwitz et al. 1995) prior to matrix division and defining the functional strengths of connections in an anatomical model. This analysis suggests an action of lithium (21 days) on the functional interaction between the amygdaloid nuclei (increased), the cingulate and frontal cortex (increased), and the thalamus and nucleus accumbens (decreased). The effects of lithium on the interregional correlation of CO activity further supported a lateralized influence on pathways of the left hemisphere. The organization of intra-amygdaloid circuitry is complex and essential for distributing an incoming stimulus so that it may be modulated by different functional systems and generate appropriate behavioral responses (Pitkanen et al. 1997). It is, thus, plausible that an action of lithium to alter processing by neural systems involving the amygdala, cingulate cortex, and nucleus accumbens could conceivably alter mood state. This study had a small sample size ($n = 6$), which may restrict the power of the correlation analysis and this estimate of functional networks related to effects of lithium administration must be considered preliminary.

In conclusion, we have refined a light microscopic, quantitative histochemical, functional neuroimaging technique for the quantification of sustained changes in neuronal activity and demonstrated that prolonged, but not short-term lithium treatment decreased activity within the cingulate cortex and nucleus accumbens. This method will be useful in identifying discrete brain areas and possible neural circuits that are altered following psychopharmacological treatment and are involved in mediating drug therapeutic and side effects.

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